Thesis Title: Characterization and interventional strategies in novel transgenic rat models of Autism spectrum disorder

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Background: Autism spectrum disorders (ASD) are neurodevelopmental disorders with repertoires of symptoms such as social interaction deficits, restrictive/repetitive behaviors and language deficits (Abrahams & Geshwind, 2008). ASD is also associated with co-morbid symptoms like intellectual disability, anxiety & epilepsy. These disorders are highly heritable, penetrant and heterogeneous with a strong genetic basis. Genomic studies in the ASD population have revealed various susceptible genes that code for synaptic proteins like cell adhesion molecules, translational regulators, ion channels, etc (Miles, 2011). In this study we have used novel transgenic rat models of synaptopathies to identify autistic-like behavioral traits. These rat models include mutation in genes coding for postsynaptic proteins like cyclin dependant kinase-like 5 (CDKL5), synaptic Ras GTPase (SYNGAP), phosphatase and tensin homolog (PTEN) and neuroligin 3 (NLGN3). We report that the rats with *Cdkl5*$^{-/y}$, *SynGAP*$^{+/-}$ and *Pten*$^{+/-}$ mutations present with social and cognitive deficits. Further, *Cdkl5*$^{-/y}$ and *SynGAP*$^{+/-}$ rats displayed high locomotor activity and deficits in motor learning in rotarod assay. Interestingly, *Nlgn3*$^{-/y}$ rats show enhanced learning and memory, motor coordination and did not display any social deficits or hyperactivity. Mutations leading to synaptopathy might have commonalities and differences with respect to their behavioral phenotypes. This study in the future will help us to investigate if these rats show shared molecular and specific neural circuit dysfunction. Additionally, the study might help in devising interventional strategies to alleviate common behavioral deficits across different synaptopathies causing ASD.

Aims & Objectives:

Behavioral characterization of transgenic rat models [Neuroligin3 (*Nlgn3*$^{-/y}$), Cyclin dependent kinase 5 (*Cdkl5*$^{-/y}$), Phosphatase and Tensin Homolog (*Pten*$^{+/-}$) and Synaptic Ras GTPase (*Syngap*$^{+/-}$)] of ASD and classifying them based on commonalities and differences in the behavioral deficits.

Materials and methods:

Animals: 2 months old male rats were used for experiments. Rats were housed in a combination of 2 WT- 2 HET/KO. Experimenters were blind to genotypes of the rats.

Genotyping: Ear clips of the rat pups were collected around PND 20 and sent to Transnetyx for genotyping.

Behavioral experiments:

Three chambered social interaction task

Sociability and social novelty was assessed in three chambered social interaction arena (Figure. 1). The apparatus is a rectangular three-chambered box with transparent polycarbonate walls. Each chamber is 19 x 45 cm with an open middle section, which allows free access to each chamber. Two identical, wired cage like containers which are large enough to hold a single animal (naive/unfamiliar), one on each side were used. Test rats were habituated (2 minutes for centre chamber +10 minutes for all chambers) to the arena without wired cage on first day and with wired cages in the right and left boxes for next 3 days. Stranger rats which were non littermates and at least 1 month younger compared to test rats were used for the task. These rats were also habituated to the wired cages only on day 1 and over 3 days with cage mates and dummy rats (age same as test rats) respectively. Testing was done on 5th day which included 2 phases. Phase 1 was to measure sociability (preference towards stranger animal vs novel object) and phase 2 was to measure social novelty (preference towards novel vs familiar rat). Each phase lasted for 12 minutes and inter phase interval was 5 minutes. The apparatus was thoroughly cleaned in between phases and trials to eliminate the odour.

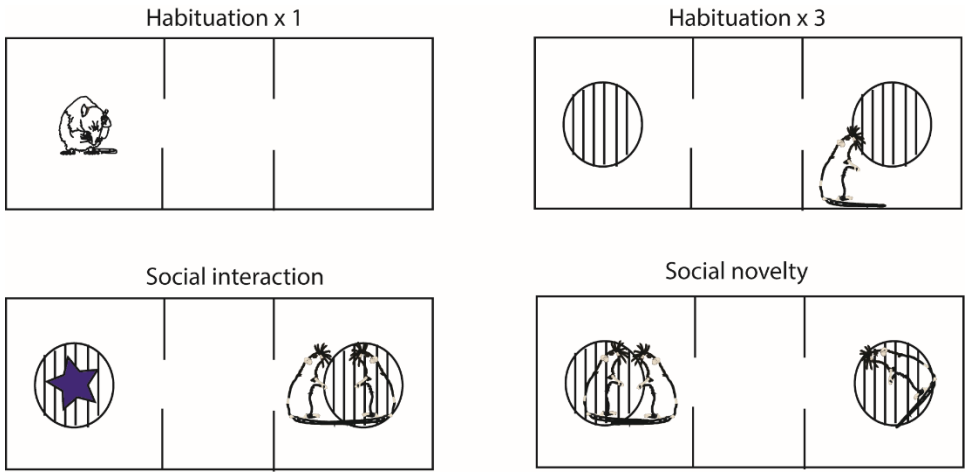


Figure.1. Schematic of three chambered social interaction task. Habituation: Day-1. B. Habituation with wired cage: Day-2-4. Social interaction phase: Day-5. Social novelty phase: Day-5.

Rotarod task

Rotarod (Columbus Instruments) was used to evaluate motor performance over 5 days (Figure. 2). The test included two phases. During the initial baseline phase, rats had to maintain their position on the spinning spindle for 90 seconds with the spindle rotating at speed of 4 RPM over 2 trials/day. 30 minutes after baseline trials the rats were subjected to the accelerating phase, where the rats had to hold their position on the spindle which accelerated at speed from 4-40 RPM (step increase of 4 RPM every 8 seconds; 5 trials/day). Latency to fall and maximum RPM attained were measured.

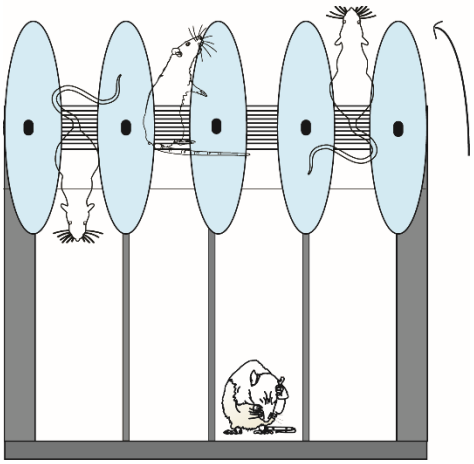


Figure.2. Schematic of Rotarod

Active place avoidance task

The methods and some of the results for this behavior in this synopsis have been published:

> Natasha J. Anstey*, **Vijayakumar Kapgal***, Shashank Tiwari, Thomas C. Watson, Anna K. H. Toft,Owen R. Dando, Felicity H. Inkpen, Paul S. Baxter, Zrinko Kozić, Adam D. Jackson, Xin He,Mohammad Sarfaraz Nawaz, Aiman Kayenaat, Aditi Bhattacharya, David J. A. Wyllie, Sumantra Chattarji, Emma R. Wood, Oliver Hardt and Peter C. Kind. Imbalance of flight–freeze responses and their cellular correlates in the Nlgn3−/y rat model of autism. *Molecular Autism*, 2022; 13:34. https://pubmed.ncbi.nlm.nih.gov/35850732/

Loco-motor activity in open field arena

Rats were allowed to explore open field (60*60) arena, 20 lux, fresh bedding (2 inch) for 10 minutes (Figure. 3). The loco-motor activity was recorded with the overhead camera and the offline analysis was done using Ethovision XT tracking and analysis software.

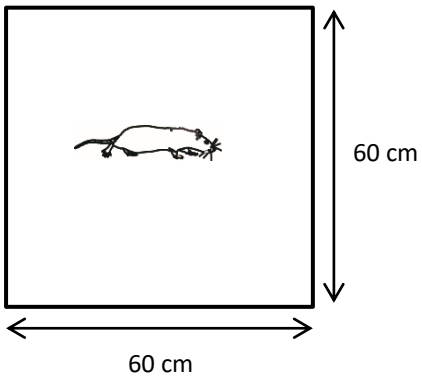
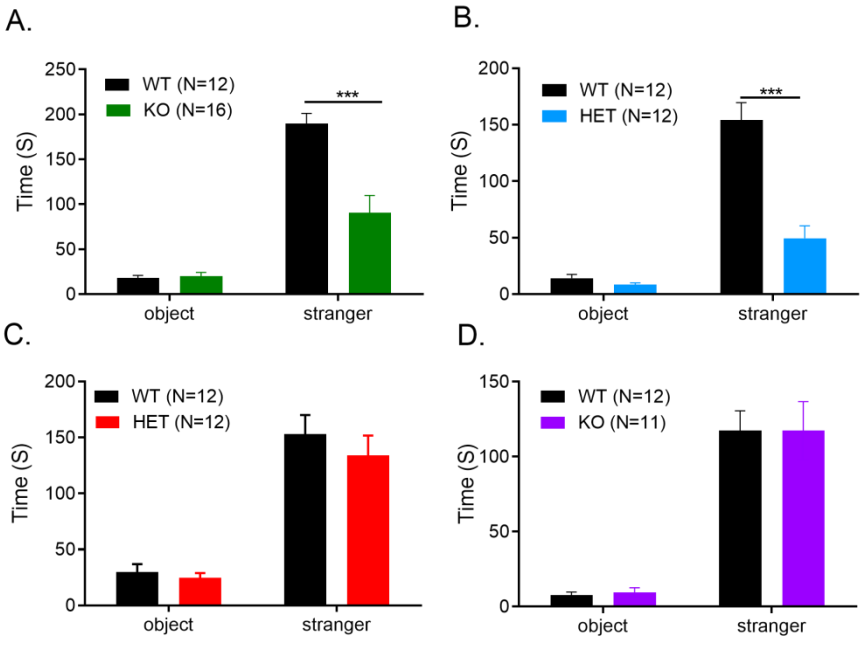


Figure.3. Schematic of open field arena

Results:

Altered social interaction in novel transgenic rat models of ASD

Social interaction deficits are definitive symptoms in ASD individuals. We assessed social interaction and social preference of these novel transgenic rats using a three chambered test. After habituation to the apparatus, rats were first assessed for sociability. Control conspecific non littermate rat was placed in one of the wired cage and a novel object was placed in another wired cage. Sociability in this assay is typically defined as more time spent in sniffing the non-familiar rat compared to the novel object (Figure. 4a). *Cdkl5*$^{-/y}$ and *SynGAP*$^{+/-}$ rats displayed reduced interaction time with the social stimulus (*Cdkl5*$^{-/y}$*:* WT-189±17.52s; KO-90.96±17.52s, *SynGAP*$^{+/-}$: WT-154.1 ± 13.64s; Het-49.62±13.64s). However, *Pten*$^{+/-}$ and *Nlgn3*$^{-/y}$ did not display any reduction in interaction with social stimulus (*Pten*$^{+/-}$: WT-153.3± 18.23s; Het-133.9±13.64s, *Nlgn3*$^{-/y}$: WT-117.6± 16.7s; KO-117.3±16.7s). Further, during the social novelty phase, test rats have to choose between the familiar conspecific non littermate rat and novel conspecific non littermate rat (Figure. 4b). Interestingly, *CDKL5*$^{-/y}$*, SynGAP*$^{+/-}$ and *Pten*$^{+/-}$ rats show reduced time spent in interaction with the novel social stimulus over familiar social stimulus (*Cdkl5*$^{-/y}$*:* WT-69±14.21s; KO-31.58±14.21s, *SynGAP*$^{+/-}$: WT-105.1 ± 17.09s; Het-10.67±17.09s; *Pten*$^{+/-}$: WT-118.1± 21.61s; Het-



58.7±21.61s). Again, *Nlgn3*$^{-/y}$ did not display any reduction in interaction with novel social stimulus (WT-69.1± 16.42; KO-64.51± 16.42s).

Figure.4a. Altered sociability in novel transgenic rat models of ASD. A & B. *CDKL5*$^{-/y}$ and *SynGAP*$^{+/-}$ rats show reduced time spent with social stimulus. C & D. *Pten*$^{+/-}$ and *Nlgn3*$^{-/y}$ rats do not show deficits in sociability. Two-way ANOVA. Sidak's multiple comparison test. *Cdkl5*$^{-/y}$: Genotype- $F_{(1,52)}$=15.26, Interaction : $F_{(1,52)}$=16.43; *SynGAP*$^{+/-}$:Genotype-$F_{(1,44)}$=32.76, Interaction: $F_{(1,44)}$=26.16; *Pten*$^{+/-}$: Genotype - $F_{(1,44)}$=0.89, Interaction : $F_{(1,44)}$=0.3; *Nlgn3*$^{-/y}$: Genotype- $F_{(1,44)}$=0.003, Interaction : $F_{(1,44)}$=0.007. p<0.0001***, p<0.001**, p<0.05*. P<0.0001***

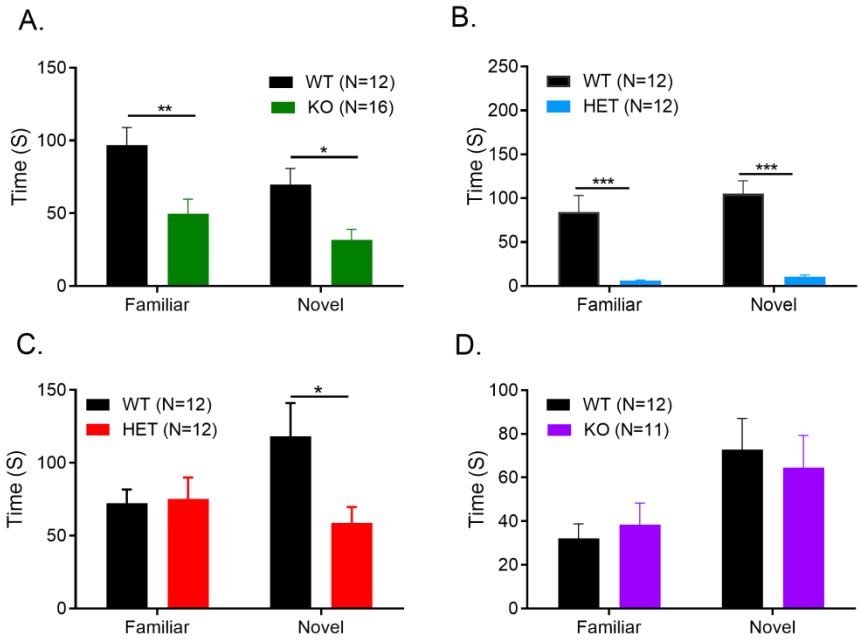


Figure.4b. Altered social novelty in transgenic rat models of ASD. A, B & C. *CDKL5*$^{-/y}$, *SynGAP*$^{+/-}$ and *Pten*$^{+/-}$ rats show reduced time spent with social stimulus. D. *Nlgn3*$^{-/y}$ rats do not show deficits in sociability. Two-way ANOVA. Sidak's multiple comparison test. *Cdkl5*$^{-/y}$: Genotype- $F_{(1,50)}$=17.95, Interaction : $F_{(1,50)}$=0.19; *SynGAP*$^{+/-}$:Genotype-$F_{(1,44)}$=5.17, Interaction: $F_{(1,44)}$=0.44; *Pten*$^{+/-}$: Genotype - $F_{(1,42)}$=3.39, Interaction : $F_{(1,42)}$=4.18; *Nlgn3*$^{-/y}$: Genotype- $F_{(1,44)}$=0.004, Interaction : $F_{(1,44)}$=0.17. p< 0.0001***, p<0.001**, p<0.05*.

Motor co-ordination in novel rat models of ASD

Motor deficits are the co-morbid symptoms which appear early in the childhood in patients with ASD. 80-90% children with ASD present with some degree of motor impairments which includes issues with gait, balance, muscle tone, strength, fine and gross motor skills etc., (Hilton et al., 2012). Previous

research has shown strong correlation between motor impairments and social & cognitive deficits (church et al., 1999 & Bruininks and Bruininks, 2005). We assessed the motor co-ordination using a rotarod test. All the four transgenic rat models of ASD showed comparable motor co-ordination to their respective control rats in the steady state phase of the rotarod test (Figure. 5a). In this phase the rats had to balance on the slower rotating spindle (4 rpm). In the accelerating phase where there is a challenge to balance on the accelerating spindle, *CDKL5*$^{-/y}$ and *SynGAP*$^{+/-}$ rats fell off early on lower speeds compared to their respective control rats (*Cdkl5*$^{-/y}$*:* WT-52.23±5.52s; KO-37.97±5.52s, *SynGAP*$^{+/-}$: WT-50.25 ± 3.12s; Het-38.72±3.12s).Hence, *CDKL5*$^{-/y}$ and *SynGAP*$^{+/-}$ rats show defcits in motor learning. *Pten*$^{+/-}$ rats displayed comparable motor learning during the accelerating phase of the rotarod test (WT-48.82 ± 4.08s; Het-45.13±4.08s). Interestingly, *Nlgn3*$^{-/y}$ rats remained for longer time on the spindle at the higher speeds compared to their control rats (WT-40.85±4.13s; KO-61.39±4.13s).Thus, displaying enhanced motor performance during accelerating phase of rotarod test (Figure.5b).

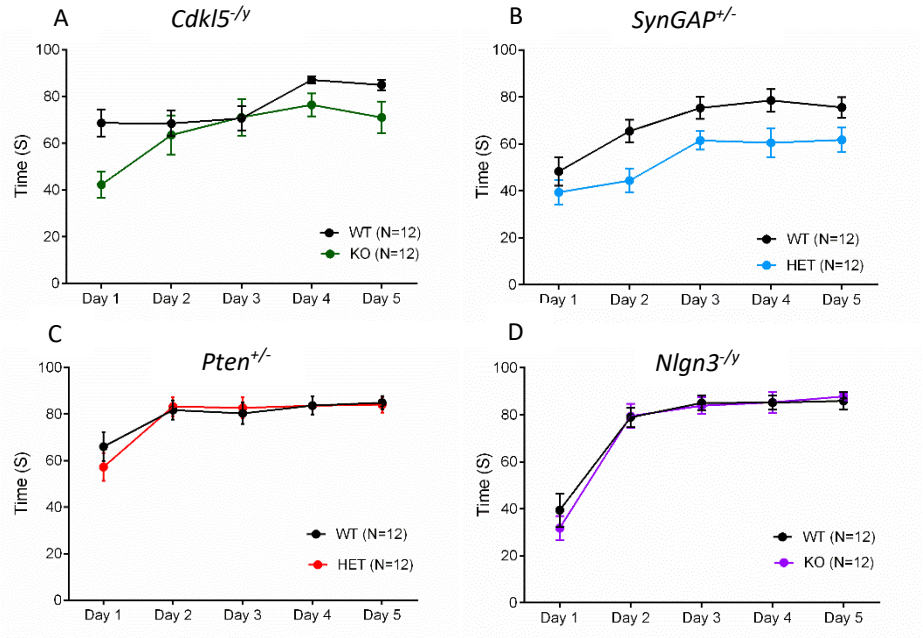


Figure 5a. Unaltered motor co-ordination in baseline phase of rotarod test. Repeated measure two way ANOVA.Post hoc test: Sidak's multiple comparison's test.

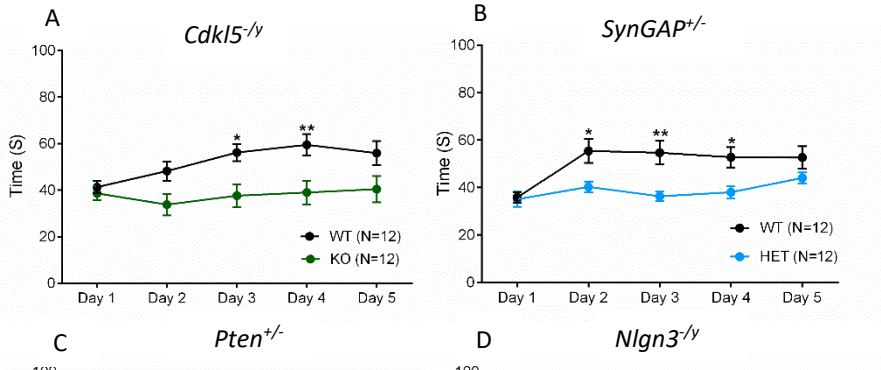
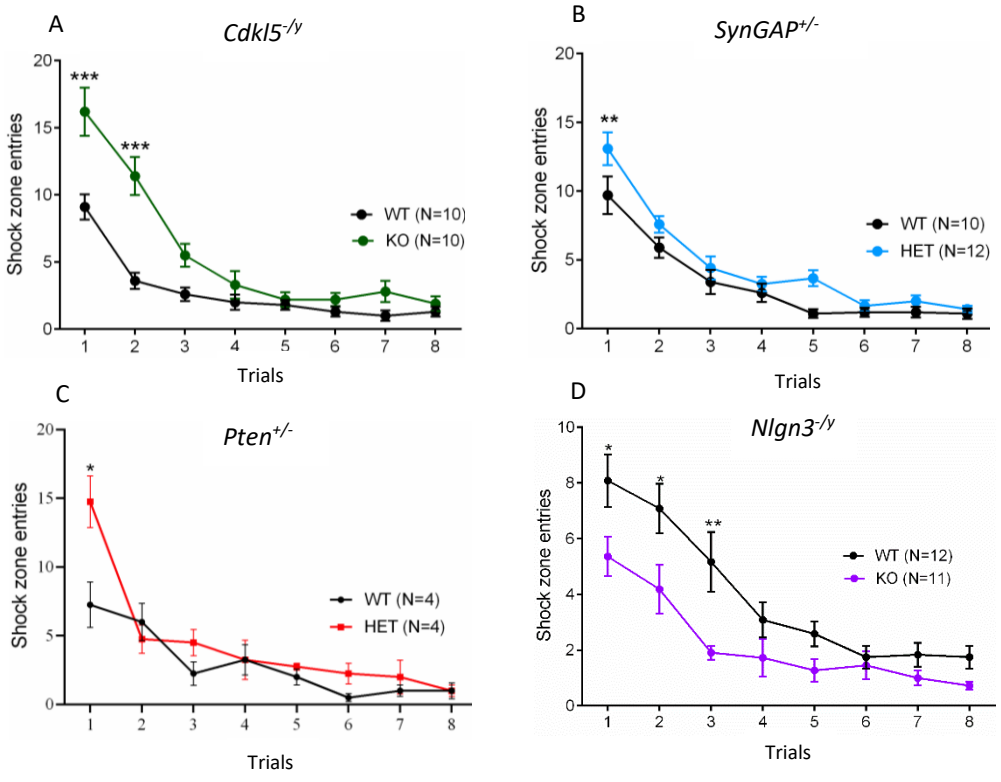


Figure.5b. Motor deficits during accelerating phase of rotarod test. Repeated measure two-way ANOVA. Post hoc test: Sidak's multiple comparison test. *Cdkl5*$^{-/y}$: Genotype- $F_{(1,28)}$=13.64, Interaction : $F_{(4,112)}$=2.73; *SynGAP*$^{+/-}$:Genotype-$F_{(1,22)}$=6.72, Interaction: $F_{(4,88)}$=4.27; *Pten*$^{+/-}$: Genotype - $F_{(1,26)}$=0.058, Interaction : $F_{(4,104)}$=0.98; *Nlgn3*$^{-/y}$: Genotype- $F_{(1,22)}$=27.63, Interaction : $F_{(4,44)}$=4.21. p<0.0001***, p<0.001**, p<0.05*.

Cognitive deficits in novel transgenic rat model of ASD

Children with ASD present with impairments in executive function which include cognitive flexibility, working memory, attention and verbal and non-verbal learning (Velikonja et al., 2019). We assessed cognitive function in these novel rat models of ASD using a spatial learning and memory task. Active place avoidance task utilizes a rotational platform with designated shock zone. Rats have to actively learn to avoid the shock zone using the 3-dimensional cues located in the experiment room. Post learning rats were given a shock less probe trial to assess the avoidance memory. In an active place avoidance task, *CDKL5*$^{-/y}$, *SynGAP*$^{+/-}$, and *Pten*$^{+/-}$ rats exhibit delayed avoidance learning. Comparative to their respective control rats, these rats entered the shock zone more frequently (*Cdkl5*$^{-/y}$*:* WT-2.8±0.59; KO-5.68±0.59, *SynGAP*$^{+/-}$: WT-3.27±0.43; Het-4.63±0.43; *Pten*$^{+/-}$: WT-2.9±0.68; Het-4.41±0.68)(Figure. 6a). Interestingly, *Nlgn3*$^{-/y}$ rats show enhanced acquisition of avoidance learning. They entered



significantly less number of times compared to their controls (WT-4.3±0.55; KO-2.6±0.55). Additionally, *Cdkl5*$^{-/y}$ and *Pten*$^{+/-}$rats displayed equivalent avoidance memory to their respective controls throughout the shock-free probe trial (*Cdkl5*$^{-/y}$: WT-1.8±0.99; KO- 1.9±0.99, *Pten*$^{+/-}$: WT-7.25± 6.75; Het-14.75± 6.75). Whereas, *SynGAP*$^{+/-}$ and *Nlgn3*$^{-/y}$ rats entered less number of times into the shock zone. Hence, exhibiting stronger avoidance memory (*SynGAP*$^{+/-}$: WT-8.5±2.05; Het- 2.92± 2.05, *Nlgn3*$^{-/y}$: WT-13.42±2.09; KO-6.54±2.09)(Figure. 6b).

Figure 6a. Learning deficits during active place avoidance test. Repeated measures two-way ANOVA. Post hoc test: Sidak's multiple comparison test. *Cdkl5*$^{-/y}$: Genotype- $F_{(1,18)}$=22.66, Interaction : $F_{(7,126)}$=7.66; *SynGAP*$^{+/-}$:Genotype-$F_{(1,20)}$=9.56, Interaction: $F_{(7,140)}$=1.39; *Pten*$^{+/-}$: Genotype - $F_{(1,\ 6)}$=4.72, Interaction : $F_{(7,42)}$=3.66; *Nlgn3*$^{-/y}$: Genotype- $F_{(1,21)}$=10.59, Interaction : $F_{(2,42)}$=4.21. p<0.0001***, p<0.001**,p<0.05*.

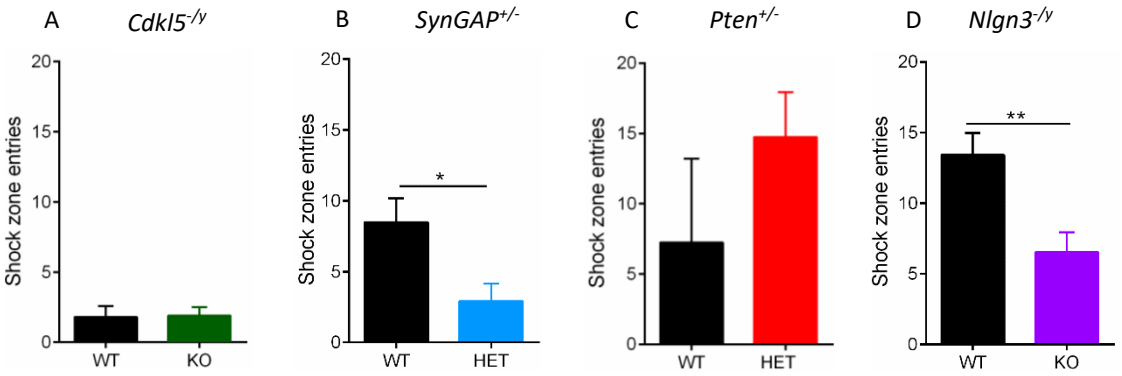


Figure.6b. Avoidance memory in novel transgenic rat models of ASD. Unpaired t-test p<0.001**, p<0.05*

Hyperactivity in novel transgenic rat models of ASD

Children with ASD present with attention deficit and hyperactivity. Hyperactivity is defined as excessive mobility which hampers their ability to concentrate and results in being impulsive and aggressive. In rodents hyperactivity is measured as total distance travelled (m) in open field. We found *CDKL5*$^{-/y}$ (WT-65.64±6.78m; KO-85.26±6.78m) and *SynGAP*$^{+/-}$ (WT-65.88±4.36m; Het- 89.36±4.36m) rats to be hyperactive in the open field. Furthermore, *Pten*$^{+/-}$ (WT-41.12±2m; Het- 44.14±2m) and *Nlgn3*$^{-/y}$ rats (WT-44.02±2.09m; KO-44.66±2.09m) did not show any hyperactivity in the open field.

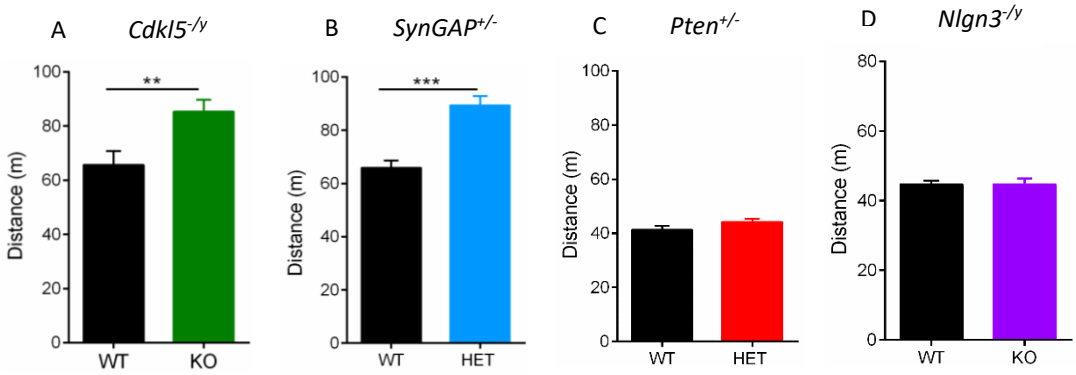


Figure. 8. Loco-motor activity in open field. Unpaired t-test $p<0.001$**, $p<0.0001$***

Discussion:

Monogenic non-syndromic autism cases have unknown etiology and recently many risk genes are being probed for their role in Autism Spectrum Disorders (ASD). Synaptopathies are major contributors to monogenic causes of ASD. Previous investigations to elucidate causal reasons behind these synaptopathies have revealed multiple mutations in genes coding for synaptic proteins. Our investigation for the first time demonstrates that the novel transgenic rat models of synaptopathies lead to autistic-like behavioral traits in *CDKL5*$^{-/y}$, *SynGAP*$^{+/-}$, *Pten*$^{+/-}$, and *Nlgn3*$^{-/y}$ rats.

Mutation in X linked gene, Cyclin dependent kinase like 5 (a serine-threonine kinase) results in severe intellectual disability and autistic features with various comorbid conditions (Szafranski et al., 2015). In our study, we observe impairments in social interaction, motor coordination, active place avoidance learning, and memory and hyperactivity in *CDKL5*$^{-/y}$ rats. CDKL5 protein has a role in dendritic growth, synaptic transmission, gene expression etc., (Zhu and Xiong, 2018). Additionally, studies have implicated CDKL5 in dendritic arborization, axon outgrowth, spine formation and synaptic transmission (Sala et al., 2016). CDKL5 is an upstream protein in the molecular cascade involving the MECP2 protein implicated in Rett's syndrome. Hence, it might be possible that these two proteins share disruption in the common neuronal circuits resulting in similar behavioral deficits. Our results are in line with mice models of CDKL5 mutations which also display similar impairment in hippocampus-dependent memory, deficits in motor coordination and social deficits (Jhang et al., 2017; Okuda et al., 2018; Amendola et al., 2014; Fuchs et al., 2014).

SynGAP haploinsufficiency results in severe mental retardation with symptoms like cognitive dysfunction, epilepsy and severe language deficits (Mignot et al., 2016). Haploinsufficiency of SynGAP leads to mental retardation and is often associated with autism (Lossifov et al., 2014, Berryer et al., 2013, Hamdan et al., 2011 & Hamdan et al., 2009). SynGAP is implicated in spine formation and maturation (Clement et al., 2012). SYNGAP is postsynaptic protein localized with post synaptic density protein complex. SynGAP plays role in regulating the *Ras* activation and AMPA receptor insertion on the postsynaptic membrane. In our study, we demonstrate that the *SynGAP*$^{+/-}$ rats show hyperactivity in the open field and impaired motor learning in rotarod. Hyperactivity could be a reason for their deficits in motor learning. *SynGAP*$^{+/-}$ rats also show reduced interest in exploring social stimulus in three-chambered social interaction task. The social deficits we observed could be either due to their lack of general interest in exploration or due to their altered synaptic transmission between brain regions responsible for social behavior. Furthermore, *SynGAP*$^{+/-}$ rats show only a transient initial delay in learning in the active avoidance task. Interestingly, we did not observe any memory deficits during the probe trial of active place avoidance task. Heterozygous SynGAP mice have also shown deficits in social interaction, learning in the Morris water maze/ Barnes maze and hyperactivity in open field (Komiyama., et al, 2002, Muhia et al., 2010, Nakajima et al., 2019 & Guo et al., 2009). However, the findings in mice models were variable and were dependent on the genetic background the mouse.

The *Pten* gene is identified as a risk gene for autism with macrocephaly (Buxbaum et al., 2007). PTEN is a negative regulator of the PI3K-AKT-mTOR pathway (Song, Salmena & Pandolfi, 2012). PTEN has a role in

cell cycle progression, cell migration, cell death, transcription and translation (Chen et al., 2018; Rademacher and Eickholt, 2019). Our study demonstrates *Pten* heterozygous rats show deficits in social novelty but not sociability. This suggests that *Pten* heterozygous rats do show normal social interaction but might have problems with social recognition memory. *Pten* heterozygous rats also display delayed acquisition of shock zone location in active place avoidance task. *Pten* heterozygous rats show milder phenotypes in social and cognitive domains. Conditional *Pten* null mice & *Pten* heterozygous mice showed reduced social interaction, learning and memory deficits. These mice also did not show hyperactivity and motor deficits (Clipperton-Allen and Page, 2014; Kwon et al., 2006). Our results corroborate with previous findings in mice.

Mutations in the synaptic protein Neuroligin-3 were originally linked to ASD by Jamain et al. (2003) and point mutations in *Nlgn3* have since been shown to be correlated with ASD/Intellectual disability (ID) by several studies (C Yuen et al., 2017). Although mutations in *Nlgn3* account for a very small proportion of ASD cases, mutations in this gene appear to be highly penetrant. NLGN3 is a postsynaptic cell adhesion molecule that binds to presynaptic neurexins. NLGN3 has a role in synapse formation, maintenance and transmission. Interestingly, we observe enhanced learning and memory in the active place avoidance paradigm and superior motor performance in the rotarod test. These phenotypes observed could be due to their exaggerated efficiency in acquiring repetitive motor routines which might help them to perform better in these behavioral tests. Evidence for this comes from a mouse study that also reported a similar motor learning phenotypes. The study associated the enhanced motor performance with a lack of inhibition in the nucleus accumbens which drives the mice to efficiently acquire repetitive motor routines in rotarod (Rothwell et al., 2014). Mice lacking NLGN3 show hyperactivity in the open field, normal sociability, learning and memory (Radyushkin et al., 2009; Jaramillo et al., 2014). A recent study in the rats have revealed that the *Nlgn3*$^{-/y}$ rats do not show sociability deficits but present with reduced juvenile play behavior (Hamilton et al., 2014).

Our study reports conserved phenotypes across species which is highly important for translational research. Further, it will be interesting to ascertain the circuit-specific behavioral dysfunction in these novel rat models. Molecular mechanisms disrupted by each of these causal gene mutations may intersect downstream along overlapping molecular pathways. A recent study has demonstrated the link between NLGN3 and PTEN via scaffolding protein Magi-2. The observed morphological phenotypes in cultured neuroligin 3 KO neurons were rescued by rapamycin treatment (Xu et al., 2019). In conclusion, novel transgenic rat models of synaptopathies reveal autism-like behavioral traits. The common or different behavioral phenotypes observed between different transgenic rats would open avenues to develop interventional strategies to alleviate the common behavioral deficits.

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